PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 35/76, A23L 3/3571, C12N 7/00, C12Q 1/70, 1/04 // (C12Q 1/04, C12R 1:92)

A1

(11) International Publication Number: **WO 98/47521**

(43) International Publication Date: 29 October 1998 (29.10.98)

(21) International Application Number: PCT/US98/07395

(22) International Filing Date: 13 April 1998 (13.04.98)

(30) Priority Data:

08/845,507	24 April 1997 (24.04.97)	US
08/994,037	18 December 1997 (18.12.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/994,037 (CON)
Filed on	Not furnished
US	08,845,507 (CON)
Filed on	Not furnished

(71) Applicant *(for all designated States except US)*: IDAHO RESEARCH FOUNDATION, INC. [US/US]; University of Idaho, Morrill Hall 103, Moscow, ID 83844–3003 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: YOUDERIAN, Philip, A. [US/US]; 2517 Idlers Rest Road, Moscow, ID 83843 (US). BOHACH, Carolyn, H. [US/US]; 300 Rose Court, Moscow, ID 83843 (US).

(74) Agents: MATKIN, Mark, S. et al.; Suite 1300, 601 West First Avenue, Spokane, WA 99201–3817 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

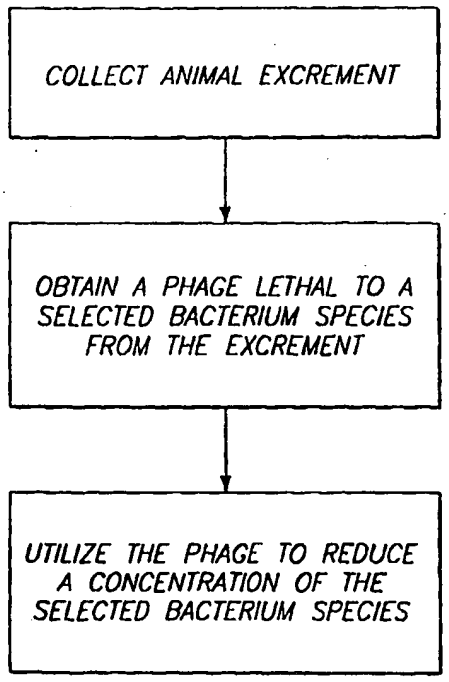
With amended claims.

With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: PHAGES, METHODS FOR GROWING AND DETECTING THEM AND THEIR USE

(57) Abstract

The invention encompasses phages, methods of growing phages, methods of utilizing phages for reducing concentrations of bacterium species pathogenic to humans, and methods for detecting bacterial strains. The invention further encompasses methods for producing meat and dairy products for human consumption. In one aspect, the invention includes a method for producing meat for human consumption comprising the following steps: a) inserting a phage lethal to a bacterium species into a digestive tract of an animal to reduce a concentration of the bacterium species within the digestive tract; and b) slaughtering the animal to produce meat for human consumption. In another aspect, the invention includes a method for producing meat for human consumption comprising the following steps: a) slaughtering an animal to produce meat; and b) applying a phage lethal to a bacterium species to the meat. In yet another aspect, the invention includes a method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract comprising the following steps: a) collecting animal excrement; b) obtaining a phage lethal to the bacterium species from the animal excrement; and c) applying the phage lethal to the bacterium species to a surface from which bacteria could contact humans to reduce a concentration of the bacterium species pathogenic to humans on said surface.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PHAGES, METHODS FOR GROWING AND DETECTING THEM AND THEIR USE

Technical Field

The invention pertains to phages, methods of growing phages, and to methods of utilizing phages for reducing concentrations of bacterium species pathogenic to humans. The invention further pertains to methods for producing plants, meat and dairy products for human consumption. Additionally, the invention pertains to methods of detecting bacterial strains.

Background Art

In 1993 an outbreak of food poisoning occurred in the Western United States. The outbreak was eventually linked to a coliform, *E. coli* O157:H7, that had been served in contaminated, under-cooked hamburger at various restaurants. In July of 1996, more than 10,000 people in Japan were stricken by a serious outbreak of food poisoning traced tentatively to daikon radish spouts contaminated by *E. coli* O157:H7, at least seven died. It is estimated that between 10,000 and 20,000 human infections with *E. coli* O157:H7 occur annually in the United States. Enterohemorrhagic strains of *E. coli* (EHEC), especially those of the serotype O157:H7, can cause human hemorrhagic colitis (bloody diarrhea). Approximately 2-7% of infections progress to hemolytic uremic syndrome, which is characterized by anemia, thrombocytopenia, kidney failure, and a death rate of 3-5%.

Unlike many pathogenic bacteria, ingestion of very few, sometimes less than 20, *E. coli* O157:H7 can cause human disease. It is thought that in the overwhelming majority of *E. coli* O157:H7 outbreaks the source of infection is contaminated ruminant (primarily bovine) food products, either under-cooked beef or unpasteurized dairy products. Disease, however, is not limited to those who eat contaminated food. For example, one of the children who died in the above-mentioned outbreak on the West Coast apparently never ate at a restaurant chain serving contaminated meat, but instead played with an infected child.

Hemolytic uremic syndrome is a dangerous disease. At the first outward signs of disease, the kidney damage is frequently already done. Even those who

receive immediate, modern medical attention are not guaranteed recovery. Accordingly, it would be desirable to develop procedures for reducing the likelihood of *E. coli* O157:H7 infection in humans. Particularly, it would be desirable to develop procedures for reducing *E. coli* O157:H7 contamination of food products. More generally, it would be desirable to develop procedures for reducing *E. coli* O157:H7 contamination on surfaces from which the bacteria may be transferred to human beings.

E. coli O157:H7 is often found in ruminant mammals. Ruminant mammals are common in our environment and comprise farm animals, such as cattle and sheep, and wild animals, such as deer. Although *E. coli* O157:H7 causes disease in humans, it is frequently not harmful to ruminant mammals. In fact, *E. coli* O157:H7 may be a common transient member of the intestinal flora of many ruminant mammals. *E. coli* O157:H7 likely also exists in the digestive tracts of other animals besides ruminant mammals. For instance, *E. coli* O157:H7 is thought to be exist in the digestive tracts of birds, including poultry, and may also exist in pigs. Additionally, *E. coli* O157:H7 appears to be substantially pervasive in the environment beyond animal digestive tracts, with unpasteurized apple juice even occasionally being contaminated with pathogenic quantities of *E. coli* O157:H7.

E. coli O157:H7 in cattle feces and intestinal tracts can find its way into humans through a number of routes. For instance, cattle can carry *E. coli* O157:H7 into meat processing plants. Once inside a meat processing plant, the bacteria can be introduced onto meat if there is contact of meat surfaces with animal feces, or with surfaces which have come in contact with animal feces. Additionally, it appears that *E. coli* O157:H7 can be transferred from an animal's haircoat, or from surfaces which have contacted an animal's haircoat. Further, it appears that *E. coli* O157:H7 can be transferred by aerosols formed, for example, when a surface having *E. coli* O157:H7 thereon is sprayed with a liquid, such as water.

If a food product becomes contaminated with *E. coli* O157:H7, detection of the contamination can be difficult. As discussed above, only very small numbers of *E. coli* O157:H7 need be ingested by a human to cause disease. Such low numbers are hard to detect. Accordingly, it would be desirable to reduce the likelihood of contamination of food products by *E. coli* O157:H7, rather than rely on detection techniques to identify contaminated food products.

It is noted that one method of ridding beef, dairy and other food products of *E. coli* O157:H7 is to heat-treat the food products to kill the bacteria. However, as it is inconvenient to fully cook all food products before ingestion, and as it is unlikely that all dairy and fruit juice products have been properly pasteurized, it would be desirable to develop alternative methods of reducing the likelihood of *E. coli* O157:H7 contamination in meat, dairy and other food products.

It is also noted that other bacteria besides *E. coli* O157:H7 cause widespread human disease. For instance, pathogenic *E. coli* O111 strains are common in New Zealand and Australia, and cause human symptoms similar to those of *E. coli* O157:H7 infection. As another example, pathogenic *Salmonella* strains are ubiquitous in the environment. Such pathogenic *Salmonella* strains include, for example, *S. enteriditis*, *S. typhi*, *S. paratyphi*, *S. dublin*, *S. cholerasuis*, and *S. pullorum*. Pathogenic *Salmonella* strains are frequently found in the digestive systems of poultry, such as chickens and turkey, and frequently cause food poisoning. It would be desirable to develop methods applicable to many types of pathogenic bacteria for reducing the likelihood of the pathogenic bacteria being ingested by humans.

Best Modes for Carrying Out the Invention and Disclosure of Invention

A block diagram of a method of the present invention is illustrated in Fig. 1. A first step of the method comprises collecting animal excrement, such as feces. The excrement may be collected by, for example, gathering manure at a farm.

The next step of the method is to obtain a phage lethal to a selected bacterium species from the animal excrement. A method of obtaining such phage is to procure a mix of phages from the animal excrement and subject the phages to growth conditions which selectively grow those which can utilize the selected bacterium species as a host. For example, phages selectively lethal toward *E. coli* O157:H7 can be obtained by the following phage detection and isolation procedure.

A culture is prepared comprising 5 mls of *E. coli* O157:H7 at an exponential growth phase. The *E. coli* is preferably in Luria broth (Luria broth is described in Molecular Cloning - a Laboratory Manual (1989) 2nd edition, edited by Sambrook J., Fritsch E.F., and Maniatis T., Cold Spring Harbor Laboratory Press, New York.) with 10 mM $MgSO_4$. The culture is inoculated

with 0.5 gm of a fecal sample. Thus, the exponentially growing culture is inoculated with a material collected from a non-sterile environment. The fecal sample can be placed directly into the exponentially growing *E. coli* without any purification occurring after collecting the fecal sample.

After the inoculation, the culture is grown for 12 hours at 37° C. The culture is then spun in a low-speed centrifuge to precipitate the bacteria while leaving the phages in a supernatant. The supernatant is treated with chloroform (about 1 part chloroform to 20 parts solution, by volume) to kill any bacteria remaining in the supernatant. Aliquots of 0.1 ml to 0.5 ml are removed from the supernatant and spread on lawns of *E. coli* O157:H7. The lawns are prepared by a soft-agar overlay technique described by Adams. (*See*, Adams, M. H. (1959) Bacteriophages, Interscience Publishers, New York.)

If a phage lethal to *E. coli* O157:H7 is present, a plaque will form. Lethal phages can be removed from the plaque and used to grow a high-titer (concentration) phage stock by methods known to persons of ordinary skill in art. Such methods can include, for example, the following process. A plug of a plaque is removed and resuspended in 1 ml of Luria broth containing 1 mM $MgSO_4$. Aliquots of 0.1 ml to 0.5 ml of the resuspended plaque material are utilized to infect cultures containing 30-300 mls of *E. coli* O157:H7 at an exponential growth phase in Luria broth containing 1 mM $MgSO_4$. The cultures are grown for 6-18 hours at 37° C and then spun in a low-speed centrifuge to precipitate the bacteria while leaving the lethal phages in a supernatant. The supernatant is treated with chloroform (about 1 part chloroform to 20 parts solution, by volume) to kill any bacteria remaining in the supernatant. The lethal phages can then be assayed on one or more non-O157 strains of *E. coli*, such as, for example, 091 strains, 05 strains and 0111 strains, to determine if the lethal phages are selectively lethal to O157 strains of *E. coli*. Phages which are selectively lethal to O157 strains of *E. coli* are preferred for removing *E. coli* O157:H7 from ruminant digestive tracts. Such phages are unlikely to disrupt ruminant digestion by destroying microbial flora other that *E. coli* O157:H7.

The phage detection and isolation procedure described above yielded the results shown in Table 1. Specifically, Table 1 shows results obtained from subjecting 53 fecal samples to the above-described procedure. The 53 samples were collected from five different farms in Idaho and Washington. As shown,

the fecal samples yielded five phages, labeled 1-5. The phages labeled 1, 3, 4, and 5 were lethal for *E. coli* O157 strains and not for 091 strains, 05 strains and 0111 strains of *E. coli*.

Table 1

SOURCE	NUMBER OF POSITIVES	PHAGE LABEL
University of Idaho Beef Farm	0/4	
University of Idaho Dairy Farm	1/8	1
Washington State University Dairy Farm	1/8	3
Private Dairy Farm	2/23	2,4
University of Idaho Sheep Pen	1/8	5

Table 2 illustrates growth patterns obtained from the phages identified as 1, 3, 4, and 5 in Table 1. The phages yielded three different patterns of growth on eight independent, different O157 isolates. The different O157 isolates were:

O157 isolate 1 = ATCC 43894

O157 isolate 2 = ATCC 43889

O157 isolate 3 = ATCC 43890

O157 isolate 4 = 549-9-LIII

O157 isolate 5 = 9024-1-LIII

O157 isolate 6 = 5019-13-LIII

O157 isolate 7 = G6001

O157 isolate 8 = 35150

The data of Table 2 reflects the relative ability of a particular phage stock to clear a lawn of each host bacterium. Phage stocks were diluted by serial 10-full dilutions and spotted on lawns of each type of indicator cells. Each "plus" indicates the number of serial 10-full dilutions that still allowed a phage stock to form clearings, or plaques, on a particular indicator lawn. The patterns are not due to the bacteria overcoming phage infection through host restriction/modification.

Table 2

	Phages 1,3	Phage 4	Phage 5
O157 isolate 1	+++	+++	+++
O157 isolate 2	+++++	++	+
O157 isolate 3	+++++	+++	++
O157 isolate 4	+++	+++	+
O157 isolate 5	+++	+++	+
O157 isolate 6	++++++	+	+
O157 isolate 7	+++	+++	+
O157 isolate 8	+++	++	+

In one embodiment of the present invention, phages, such as those described with reference to Tables 1 and 2, are further subjected to conditions selective for growth at 50° C to isolate temperature-stable, spontaneous mutants of the phages. For instance, the phage labeled 4 in Table 1 was temperature-treated at 50° C by the following procedure. The phage, in Luria broth with 10 mM $MgSO_4$, was maintained at 50 °C for two hours. Subsequently, the phage was inoculated into a culture of exponentially growing *E. coli* O157:H7, in Luria broth containing 10 mM $MgSO_4$, and grown for 6-18 hours in a procedure similar to that described above for isolating phages lethal to *E. coli* O157:H7. Phages were removed from the *E. coli* O157:H7 culture by spinning the bacteria from the culture and applying chloroform to the supernatant to kill any remaining bacteria. The phages were then resubjected to temperature treatment at 50 °C for 2 hours. The processes of temperature treatment and

growth of the phages were repeated until the phages had been subjected to four total temperature treatments at 50 °C for 2 hours each. Such processing yielded a temperature-stable phage referred to in our labs as ER4hr1, which has been deposited at the American Type Culture Collection (ATCC), at 12301 Parklawn Drive, Rockville, MD 20852, U.S.A., and is identified by the ATCC No. 55952. It is thought that the above-discussed procedure selectively grows a pre-existing phage that is temperature stable, rather than inducing mutations in the phages to create a temperature-stable phage. In other words, it is thought that the phages labeled 1-5 in Table 1 are actually mixtures of various naturally-occurring, very similar phages, some of which are temperature stable and some of which are not.

The temperature-stable phage ER4hr1 is substantially fully stable to exposure to a temperature of 50 °C for 2 hours. This in sharp contrast to the phage 4 of Table 1 from which ER4hr1 was derived. Phage 4 would typically lose about 90% of its activity after 2 hours of exposure to a temperature of 50 °C.

It has been found that the temperature-stable phage ER4hr1 is also stable for relatively prolonged exposure to pH extremes of 0 to 11. Whereas phage 4 would lose about 90% of its activity after 2 hours of exposure to a pH of either 0 or 11, ER4hr1 loses almost no activity during the same amount of exposure to the same pH's.

The pH and temperature stability of ER4hr1 could advantageously prolong the useful life of the phage in an animal's digestive tract. Also the pH and temperature stability of ER4hr1 could advantageously extend the available methods for transporting ER4hr1. For instance, ER4hr1 may better withstand the temperature extremes of unrefrigerated rail-car or truck transport than a phage that had not been temperature treated. Specifically, phages which are temperature treated may be able to better withstand storage at temperatures of 30° C or higher for longer periods than phages which are not temperature treated.

Although the above temperature selection procedure was described for a temperature of 50°C, it will be recognized by persons of ordinary skill in the art that a similar procedure can be utilized for a wide range of temperatures, including, for example, temperatures of 25°C and above.

Referring again to Fig. 1, the phages obtained from animal excrement are utilized to reduce a concentration of a bacterium species. Methods by which the phages may be utilized to reduce a concentration of a bacterium species include, for example, applying the phages to surfaces to kill bacterium species on such surfaces. As another example, the phages may be inserted into an animal's digestive tract to reduce a concentration of a bacterium species within the digestive tract. Particular embodiments of the present invention are discussed with reference to Figs. 2-5.

Referring to Fig. 2, a mass 10 comprising a phage is fed to an animal 12 by a handler 14. Mass 10 could comprise, for example, a liquid-filled capsule comprising phages in a liquid medium, or a solid comprising desiccated phages. If the phages are in a liquid medium, the phages can be in, for example, a buffered saline solution comprising about 10 mM $MgSO_4$.

In one aspect of the invention, animal 12 is a ruminant, such as a cow, and mass 10 comprises phages selectively lethal to *E. coli* O157:H7, such as the phage identified in our lab as ER4hr1, and identified by ATCC No. 55952. Ingestion of mass 10 by animal 12 inserts the phages into a digestive tract of animal 12. In applications in which the phages are selectively lethal to *E. coli* O157:H7, the phages will reduce the concentration of *E. coli* O157:H7 within the digestive tract of animal 12 without significantly reducing concentrations of other bacteria within the animal's digestive tract. This can advantageously rid an animal of *E. coli* O157:H7 without adversely affecting the animal's digestive processes.

Mass 10 can comprise a controlled dosage of a phage. Alternative methods for administering a controlled dosage of a phage to an animal are recognized by persons of ordinary skill in the art, and would include, for example, pouring a dose of a liquid into the mouth of animal 12. As will be recognized by persons of ordinary skill in the art, a phage could be inserted into a digestive tract of animal 12 by methods other than administering a controlled dosage to animal 12.

An alternative method of inserting phages into an animal's digestive tract is illustrated in Figs. 3 and 4. Referring first to Fig. 3, a phage solution 40 is applied to an animal's feed 42. Phage solution 40 will preferably comprise a buffered saline solution, and will more preferably comprise a buffered saline solution containing about 10 mM $MgSO_4$. Alternative methods for applying

phages to feed 42 will be recognized by persons of ordinary skill in the art and include, for example, dusting freeze-dried phages onto the feed. Although the phage is shown applied only to a top surface of feed 42, the invention encompasses methods wherein the phage is mixed into feed 42 during or after application to the feed.

Referring next to Fig. 4, feed 42 is ingested by an animal 44, thus inserting phages into animal 44.

A method of the present invention was reduced to practice in an experiment summarized in Table 3. In the experiment, a phage obtained from cattle (genus Bos) feces was utilized to reduce *E. coli* O157:H7 concentrations in a digestive tract of a sheep (genus Ovis). The experiment comprised infecting two healthy year-old Suffolk ewes with 4×10^{10} *E. coli* O157:H7 strain ATCC 43894 utilizing a procedure described by Kudva et. al. (*See*, Kudva I. T., Hatfield, P. G., and Hovde C. J. (1995) Applied and Environmental Micro. Vol. 61, pg. 1362-1370.) Specifically, the ewes were orally inoculated with a 20 ml overnight culture of ATCC 43894 in Luria broth using a 20 ml sterile syringe cartridge (no needle). One of the ewes was designated an experimental animal and the other a control animal. Two days after infecting the ewes with *E. coli* O157:H7, the experimental animal was given a dose of 2×10^9 of the phage ER4hr1 suspended in 20 ml of Luria broth with 10 mM $MgSO_4$, and the other ewe was given Luria broth without phage. The two ewes were maintained on an alfalfa-hay diet throughout the course of the experiment and penned separately.

Table 3

Control Animal O157:H7/ 0.2 g feces				Experimental Animal O157:H7/ 0.2 g feces		
Day	Not Enriched	Enriched	Phage/ gm	Not Enriched	Enriched	Phage/ gm
1	9×10^4	3×10^7	0	2×10^3	2×10^7	0
2	2×10^4	3×10^7	0	2×10^3	2×10^7	0
3	6×10^4	6×10^7	0	5×10^4	1×10^8	0
4	2×10^4	2×10^7	0	8×10^3	7×10^8	1×10^8
5	1×10^2	7×10^7	0	4×10^3	3×10^7	4×10^7
6	1×10^2	4×10^6	0	7×10^1	8×10^7	5×10^7
7	4×10^2	1×10^7	0	0	0	2×10^8
8	0	9×10^6	0	0	0	3×10^8
9	0	2×10^5	0	0	0	3×10^6
10	0	4×10^4	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0

Fecal samples (10 gm) were taken at 24 hour intervals for 12 days from both the control animal and the experimental animal. The fecal samples were assayed for the presence of *E. coli* O157:H7 before and after enrichment for *E. coli* O157:H7, utilizing a procedure described by Kudva et. al. Also, the fecal samples from the experimental animal were assayed for the presence of phage ER4hr1. The procedure utilized for assaying for ER4hr1 was as follows. Feces (1 gm) were suspended in 10 ml of Luria broth containing 10 mM $MgSO_4$ with a vortex mixer. The resuspended feces were maintained at 40 °C for 2-4 hours, then spun in a low speed centrifuge to pellet the bacteria and particulate matter, and form a supernatant. Chloroform was added to the supernatant and the supernatant was then filtered through a 0.45 micron filter. About 0.5 ml of the supernatant was plated on a lawn of *E. coli* O157:H7 to quantitate the amount of phage present.

Referring to Table 3, Day 0 corresponds to the day on which *E. coli* were fed to the sheep, and Day 2 corresponds to the day on which phages were

fed to the experimental animal. The data shows that the experimental animal cleared *E. coli* O157:H7 from its digestive tract four days earlier than the control animal cleared *E. coli* O157:H7 from its digestive tract. Clearance of *E. coli* O157:H7 from the experimental animal was concomitant with the appearance of phage in the fecal material of the experimental animal, indicating that the phages were responsible for the clearance of *E. coli* O157:H7. Also, more phages came out of the experimental animal than had been put into the experimental animal, indicating that the phages had multiplied within the experimental animal.

It is noted that the abundance of total coliforms per gram of fecal material remained substantially constant throughout the course of the experiment for both animals (data not shown), indicating that the phage ER4hr1 is substantially selectively lethal for *E. coli* O157 strains. In other words, indicating that the phage ER4hr1 is lethal for *E. coli* O157 strains and non-lethal for other coliforms.

It is also noted that by Day 11 even the control animal had shed the *E. coli* O157:H7 from its system. This evidences that ruminants naturally shed *E. coli* from their system, a phenomenon known to persons of ordinary skill in the art. However, ruminants also typically get reinfected with *E. coli*, such as with *E. coli* O157:H7. The source of reinfection is typically unknown.

As *E. coli* O157 strains are naturally cleared from animal systems and then reestablished in the animal systems, phages specific for *E. coli* O157 strains are expected to also be cleared from animal systems after a period of time. Accordingly, such phages may have to be reintroduced into animal digestive tracts at regular intervals to keep the animals clear of *E. coli* O157 strains. Alternatively, animals could be cleared of *E. coli* O157 strains and at the same time the animals' pens could be cleared of *E. coli* O157 by applying phages within the animals' pens. Alternatively, as the most danger of *E. coli* contamination of animal meat is likely at slaughter, it may be most preferable to insert phages into animal digestive tracts a few days prior to slaughter.

The experiment summarized in Table 3 shows that fecal samples obtained from an animal of a first genus can be utilized to obtain phages for insertion into an animal of a second, different genus. Thus, the present invention encompasses methods in which phages are obtained, for example, from fecal samples of an animal which is to have phages inserted within it; from fecal

samples of an animal of the same genus as the animal which is to have phages inserted within it; or from fecal samples of an animal of a different genus than the animal which is to have phages inserted within it.

Another embodiment of the present invention is described with reference to Fig. 5. Fig. 5 illustrates an animal carcass 20 being sprayed with a phage solution 22 by a worker 24 in a meat processing plant. Phage solution 22 is preferably lethal to a bacterium species pathogenic to humans. Application of phage solution 22 to surfaces within the meat processing plant, including animal carcass 20 and surfaces with which animal carcass 20 may come in contact, can reduce a risk of human ingestion of the pathogenic bacterium species. If the phage and a bacterium pathogenic to humans come into proximity with one another, the phage can infect and kill the bacterium. The phage solution can be applied to surfaces either before or after pathogenic bacterium species contact the surface. Also, a phage applied to a surface may leave the surface and ultimately contact a bacterium pathogenic to humans someplace other than the surface.

In a particular aspect of the present invention, phage solution 22 comprises a phage specific for *E. coli* O157:H7, such as the phage ER4hr1, identified by the ATCC No. 55952. Application of phage ER4hr1 to meat surfaces and to surfaces with which the meat may come in contact can reduce a likelihood of the meat leaving the processing plant contaminated with *E. coli* O157:H7. Application of the phage ER4hr1 can thus reduce a likelihood of human ingestion of pathogenic *E. coli* O157 strains.

It is noted that the present invention encompasses methods beyond those shown in Figs. 2-5. For instance, the method of the present invention encompasses general methods of reducing concentrations of bacterium species on human food items by applying phages lethal to such bacterium species onto surfaces of the food items. Additionally, the present invention encompasses methods for producing dairy products for human consumption in which an animal is milked to produce a dairy product, and phages lethal to a bacterium species are applied to the dairy product. Such methods encompass application of phages to a dairy product to reduce concentrations of pathogenic *E. coli* 0157 strains within the dairy product. The phages could comprise, for example, ER4hr1.

The invention further encompasses methods for producing meat for human consumption. Such methods can include inserting a phage lethal to a pathogenic

bacterium species into a digestive tract of an animal prior to slaughtering the animal. The methods can further include, after slaughtering the animal, applying the phage to the meat obtained from slaughter to help insure that the meat is not contaminated with pathogenic bacterium species introduced onto the meat through mishandling during or after slaughter. Although the animals shown in Figs. 2, 3 and 5 are ruminants, it is to be understood that the method of the present invention can be used for treating any animal, including, for example, swine and poultry.

The invention further encompasses methods of utilizing phages for reducing pathogenic *Salmonella* contamination of food products, such as, for example, poultry products. Such phages could be obtained from, for example, poultry manure.

The invention encompasses methods of utilizing phages for reducing pathogenic bacterial contamination of plant products, such as, for example, fruit and vegetable products. An example fruit product is apple juice. An example vegetable product is lettuce leaves.

Experiments have been conducted to demonstrate the applicability of a method of the present invention for reducing bacterial contamination of plant products. Specifically, leaf lettuce was inoculated with *E. coli* O157:H7 by submerging the leaves in a saline solution having 10^5 colony forming units (cfu) of *E. coli* O157:H7 per ml. The leaf lettuce was incubated in the saline solution for 10 minutes, which resulted in approximately 10^5 cfu being deposited per gram of lettuce. Lettuce leaves were removed from the saline solution and placed in either SM (Suspension Medium comprising 0.1 M NaCl; 0.01 M $Mg(SO_4)$; 2% gelatin; and 1 M Tris-HCl, pH 7.5) without phage (control) or SM with phage (experiment). The SM with phage contained 10^7 plaque-forming units (pfu) of the O157-specific phage ER4hr1. After 6 hours of incubation, the lettuce leaves incubated with phage had 10-fold less *E. coli* O157:H7 than did the control lettuce leaves. In addition, 1000 times less *E. coli* O157:H7 was present in the SM having phage than in the control SM. These experimental findings support the concept that phages can be utilized to reduce bacterial contamination of plant products.

The invention further encompasses methods of utilizing phages for specific identification of bacterial pathogens. For instance, as the phages labeled 1, 3, 4 and 5 in Table 1 are relatively specific for utilizing *E. coli* O157 strains as

hosts, such phages may be used to identify the presence of *E. coli* O157 strains. ER4hr1 is also relatively specific for utilizing *E. coli* O157 strains as hosts, and can therefore also be utilized to identify *E. coli* O157:H7 strains.

An example method for utilizing an phage specific for an *E. coli* strain to identify the *E. coli* strain is described by Bonner and Cliver. (*See*, Bonner A. B. and Cliver D. O. (1990) Isolation and Characterization of a Coliphage Specific for Escherichia coli O157:H7, Journal of Food Protection, pgs. 944-947.)

The method of Bonner and Cliver is an *in vitro* method for detecting *E. coli* O157:H7. The present invention encompasses an *in vivo* method for detecting *E. coli* O157:H7. Specifically, the invention encompasses a method wherein a phage specific for *E. coli* O157:H7 is inserted into an animal's digestive tract and the animal's feces are subsequently monitored to determine if the phage multiplies within the animal. If the phage multiplies, more phage will come out of the animal then was inserted into the animal. If the phage is specific for *E. coli* O157:H7, such multiplication of the phage within the animal will indicate the presence of *E. coli* O157:H7 within the animal.

An advantage of the *in vivo* method of the present invention over the *in vitro* method of Bonner and Cliver is that the method of the present invention can eradicate *E. coli* O157:H7 from an animal while the method is being utilized to detect *E. coli* O157:H7. Thus, the method can provide information that an animal is carrying *E. coli* O157:H7 and, in a common step, reduce a concentration of *E. coli* O157:H7 within the animal.

The above-described method of *in vivo* detection can be utilized for other pathogens in addition to *E. coli* O157:H7. For instance, the method can be utilized for detection of *E. coli* O111 strains, and *Salmonella* strains, including, for example, *S. enteritidis*, *S. typhi*, *S. paratyphi*, *S. dublin*, *S. cholerasuis*, and *S. pullorum*.

The invention further encompasses methods in which phages are utilized in increasingly high concentrations to compensate for natural selection of bacteria resistant to the phages. For example, a phage could be administered to an animal at a first dose to reduce a concentration of a bacterial strain within the animal. Subsequently, the phage could be readministered to the animal at a second dose to reduce a concentration of the bacterial strain that may have re-established in the animal. The second dose could be at the same concentration as the first dose, a lower concentration than the first dose, or a higher

concentration than the first dose. Preferably, if there is evidence that the bacterial strain has acquired resistance to the phage the second dose will be at a higher concentration than the first dose. The doses of phage could comprises mixtures, either solid or liquid, containing the phage at desired concentrations.

The invention further encompasses methods in which two or more genetically different phages are utilized, either sequentially or simultaneously, to reduce concentrations of one or more pathogenic bacteria. For instance, at least two genetically different phages selective for *E. coli* O157:H7 could be mixed to form a phage cocktail. The genetically different phages could comprise, for instance, two of the phages labeled 1, 4 or 5 in tables 1 and 2. (The phage labeled 3 was not included in the list of genetically different phages because the phage labeled 3 showed the same growth patterns as the phage labeled 1 in the experiment summarized in Table 2. Accordingly, is not clear if the phages labeled 1 and 3 are genetically different from one another.) The phage cocktail could then be inserted within an animal's digestive tract to substantially simultaneously insert the at least two different phages. Alternatively, the at least two different phages could be inserted sequentially, with one of the phages being inserted first, a period of time passing, and then another of the phages being inserted. Two or more different phages could also be applied, either simultaneously or sequentially, to one or more surfaces to reduce a concentration of a bacterium species pathogenic to humans. For instance, two or more different phages could be applied to one or more surfaces in a meat processing plant.

Utilization of two or more different phages in a method for reducing a concentration of a bacterial strain can have advantages over methods which utilize only one phage. For instance, bacterial strains are less likely to develop resistance to a cocktail comprising two or more different phages then to develop resistance to a single phage. Further, if two or more different phages are applied sequentially, rather than simultaneously, a later-applied phage can remain lethal to bacteria which have developed a resistance to an earlier-applied phage.

It is noted that phages are frequently relatively unstable to prolonged exposure to ultraviolet light. Accordingly, the invention encompasses methods of protecting phages from ultraviolet light prior to, and during, utilization of the phages to destroy pathogenic bacteria. Methods for protecting the phages from ultraviolet light can include, for example, storing the phages in containers which

are relatively ultraviolet-light-impermeable, applying the phages to animal feed in darkened areas, and incorporating the phages within capsules having relatively ultraviolet-light-impermeable outer casings.

CLAIMS

1. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising inserting a phage lethal to the bacterium species into an animal's digestive tract.

2. The method of claim 1 wherein the animal is a non-human animal.

3. The method of claim 1 wherein the phage is inserted into the animal's digestive tract by applying the phage to a feed which is thereafter ingested by the animal.

4. The method of claim 1 wherein the phage is inserted into the animal's digestive tract by administering the phage to the animal as a controlled dosage.

5. The method of claim 1 wherein the animal is a ruminant.

6. The method of claim 1 wherein the animal is a bird.

7. The method of claim 1 wherein the animal is a pig.

8. A method of reducing a concentration of a bacterium species within an animal's digestive tract comprising:

collecting a sample of naturally-occurring phages;

obtaining a phage lethal to the undesired bacterium species from the sample; and

inserting the phage within the animal's digestive tract.

9. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising the following steps:

collecting animal excrement;

obtaining a phage lethal to the bacterium species from the animal excrement; and

inserting the phage into a digestive tract of an animal to reduce a concentration of the bacterium species within the digestive tract.

10. The method of claim 9 wherein the phage lethal to the bacterium species is lethal to the bacterium species and non-lethal to other bacteria which naturally occur within the digestive tract and wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement;

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host; and

testing the phages which can utilize the bacterium species as a host to determine which of the phages which can utilize the bacterium species as a host are lethal to the bacterium species and also non-lethal to other bacteria which can naturally occur within the digestive tract of the animal.

11. The method of claim 10 wherein the phage is non-lethal to all bacteria which naturally occur within the digestive tract and which are not the bacterium species pathogenic to humans.

12. The method of claim 9 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures above 25° C.

13. The method of claim 9 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures of at least 50° C.

14. The method of claim 9 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement;

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures of at least 50° C; and

wherein the method further comprises, before inserting the phage into the digestive tract, storing the phage for some period of time at a temperature of 30° C or higher.

15. The method of claim 9 wherein the animal from which the excrement is collected is the same as the animal into which the phage is inserted.

16. The method of claim 9 wherein the excrement is collected from a first animal and inserted into a second animal, the first and second animals belonging to a common genus.

17. The method of claim 9 wherein the excrement is collected from a first animal and inserted into a second animal, the first and second animals belonging to different genera.

18. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising inserting two genetically different phages lethal to the bacterium species into an animal's digestive tract.

19. The method of claim 18 wherein the two different phages are inserted substantially simultaneously.

20. The method of claim 18 wherein the two different phages are inserted sequentially.

21. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising:

inserting a first mixture into an animal's digestive tract, the first mixture comprising a first concentration of a phage lethal to the bacterium species; and

after inserting the first mixture into the animal's digestive tract, inserting a second mixture into the animal's digestive tract, the second mixture comprising a second concentration of the phage lethal to the bacterium species, the second concentration being greater than the first concentration.

22. A method for reducing a concentration of a bacterium species pathogenic to humans comprising:

applying a phage lethal to the bacterium species on a surface; and

providing a phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage.

23. The method of claim 22 further comprising, before applying the phage on the surface, obtaining the phage from an excrement of an animal.

24. The method of claim 22 further comprising, before applying the phage on the surface, obtaining the phage from an excrement of a non-human animal.

25. The method of claim 22 wherein the surface comprises meat and wherein the bacterium species is *E. coli* O157:H7.

26. The method of claim 22 wherein the surface comprises a plant surface and wherein the bacterium species is *E. coli* O157:H7.

27. The method of claim 22 wherein the surface comprises an edible surface of a plant.

28. The method of claim 22 wherein the surface comprises an edible surface of a plant and wherein the bacterium species is *E. coli* O157:H7.

29. The method of claim 22 wherein the surface comprises a lettuce leaf and wherein the bacterium species is *E. coli* O157:H7.

30. A method for reducing a concentration of a bacterium species within a meat processing plant comprising applying a phage lethal to the bacterium species to at least one surface within the meat processing plant.

31. A method for reducing a concentration of a bacterium species on a human food item comprising applying a phage lethal to the bacterium species to a surface of the food item.

32. The method of claim 1, 9, 22, 30 or 31 wherein the bacterium species is a pathogenic coliform.

33. The method of claim 1, 9, 30 or 31 wherein the bacterium species is a pathogenic form of *Salmonella*.

34. The method of claim 31 wherein the food item comprises a plant.

35. The method of claim 31 wherein the food item comprises a vegetable.

36. The method of claim 31 wherein the food item comprises a vegetable, the bacterium species is *E. coli* O157:H7, and the phage is that identified by ATCC number 55952.

37. The method of claim 31 wherein the food item comprises meat, the bacterium species is *E. coli* O157:H7, and the phage is that identified by ATCC number 55952.

38. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract comprising the following steps:

collecting animal excrement;

obtaining a phage lethal to the bacterium species from the animal excrement;

applying the phage lethal to the bacterium species to a surface; and

providing a phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

39. The method of claim 9 or 38 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host.

40. The method of claim 38 wherein said surface is on a human food.

41. The method of claim 38 wherein the step of applying the phage to said surface comprises spraying the phage on meat and other surfaces within a meat processing plant.

42. The method of claim 38 wherein the animal is a ruminant, the bacterium species is *E. coli* O157:H7, and the isolated phage is that identified by ATCC number 55952.

43. A method for selectively reducing a concentration of a bacterium species comprising the following steps:

obtaining a first phage lethal to the bacterium species;

obtaining a second phage lethal to the bacterium species, the second phage being genetically different than the first phage;

applying the first phage lethal to the bacterium species and the second phage lethal to the bacterium species to a surface; and

providing at least one of the first or second phage from the surface and a bacterium of the bacterium species in proximity to each other to infect the bacterium with the at least one of the first or second phage and to reduce a concentration of the bacterium species.

44. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract comprising the following steps:

collecting animal excrement;

obtaining a first phage lethal to the bacterium species from the animal excrement;

obtaining a second phage lethal to the bacterium species from the animal excrement, the second phage being genetically different than the first phage;

applying the first phage lethal to the bacterium species to a surface;

providing a first phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the first phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface;

applying the second phage lethal to the bacterium species to the surface; and

providing a second phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the second phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

45. The method of claim 44 wherein the first phage and the second phage are applied substantially simultaneously to the surface.

46. The method of claim 44 wherein the second phage is applied to the surface after the first phage is applied to the surface.

47. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract comprising the following steps:

collecting animal excrement;

obtaining a phage lethal to the bacterium species from the animal excrement;

applying the first phage lethal to the bacterium species to a surface, the phage being applied to a first concentration;

after applying the phage to a first concentration, reapplying the phage to the surface to a second concentration, the second concentration being greater than the first concentration; and

providing a phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

48. A method for producing meat for human consumption comprising the following steps:

inserting a phage lethal to a bacterium species into a digestive tract of an animal to reduce a concentration of the bacterium species within the digestive tract; and

slaughtering the animal and producing meat therefrom for human consumption.

49. The method of claim 48 further comprising, after slaughtering the animal, applying the phage lethal to the bacterium species to the meat.

50. The method of claim 9 or 48 wherein the animal is a ruminant and the bacterium species is a pathogenic coliform.

51. The method of claim 9 or 48 wherein the animal is a bird and the bacterium species is a pathogenic form of *Salmonella*.

52. A method for producing meat for human consumption comprising the following steps:

slaughtering an animal and producing meat therefrom; and

applying a phage lethal to a bacterium species to the meat.

53. The method of claim 38 or 52 wherein the animal is a ruminant and the bacterium species is a pathogenic strain of *E. coli* O157.

54. A method for producing dairy products for human consumption comprising the following steps:

milking an animal to produce a dairy product; and

applying a phage lethal to a bacterium species to the dairy product.

55. The method of claim 1, 9, 22, 30, 31, or 54 wherein the bacterium species is *E. coli* O157:H7 and wherein the phage is that identified by ATCC number 55952.

56. A method for producing plant products for human consumption comprising applying a phage lethal to a bacterium species to the plant products.

57. The method of claim 48, 52, 54, or 56 further comprising:

collecting animal excrement; and

obtaining the phage lethal to the bacterium species from the excrement.

58. The method of claim 56 wherein the plant products are fruit products.

59. The method of claim 56 wherein the plant products are vegetable products.

60. The method of claim 56 wherein the plant products comprise lettuce leaves.

61. A method for selectively reducing a concentration of a bacterium species within a first animal digestive tract comprising the following steps:

obtaining excrement from a second animal, the second animal being a different genus than the first animal;

obtaining a phage lethal to the bacterium species from the excrement; and

providing the phage lethal to the bacterium species within the digestive tract of the first animal to reduce the concentration of the bacterium species within the first animal digestive tract.

62. The method of claim 61 wherein the first animal is a cow and the second animal is a sheep.

63. A method of growing phage comprising the following steps:

growing a culture of a host bacteria species to an exponential growth phase; and

while the culture of host bacteria species is at the exponential growth phase, inoculating the culture with an inoculant comprising an inoculating phage.

64. The method of claim 63 wherein the inoculating phage is in a substantially purified form.

65. The method of claim 63 wherein the inoculating phage is in a substantially unpurified form.

66. The method of claim 63 further comprising:

prior to inoculating, purifying the inoculating phage from a contaminant.

67. The method of claim 63 wherein the inoculant comprises feces.

68. A method of detecting a bacterial strain *in vivo* within an animal comprising:

obtaining a phage which selectively utilizes the bacterial strain as a host;

inserting the phage into the digestive tract of an animal; and

determining if the phage multiplies within the animal.

69. The method of claim 68 wherein the obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host.

70. The method of claim 68 wherein the determining if the phage multiplies within the animal comprises determining an amount of phage excreted by the animal.

71. The method of claim 68 wherein the bacterial strain is *E. coli* O157:H7.

72. A method of detecting a bacterial strain *in vivo* within an animal which comprises, in a common step, both detecting the bacterial strain and reducing a concentration of the bacterial strain within the animal.

73. A substantially purified phage identified as ER4hr1 and by ATCC number 55952.

AMENDED CLAIMS

[received by the International Bureau on 28 September 1998 (28.09.98);
original claims 1-73 replaced by amended claims 1-45 (7 pages)]

1. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising inserting a phage lethal to the bacterium species into a non-human animal's digestive tract, the bacterium species being non-pathogenic to the non-human animal.

2. The method of claim 1 wherein the phage is inserted into the animal's digestive tract by applying the phage to a feed which is thereafter ingested by the animal.

3. The method of claim 1 wherein the phage is inserted into the animal's digestive tract by administering the phage to the animal as a controlled dosage.

4. The method of claim 1 wherein the animal is a ruminant.

5. The method of claim 1 wherein the animal is a bird.

6. The method of claim 1 wherein the animal is a pig.

7. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising the following steps:

collecting excrement from an animal;

obtaining a phage lethal to the bacterium species from the excrement;

inserting the phage into a digestive tract of a non-human animal to reduce a concentration of the bacterium species within the digestive tract of the non-human animal; and

wherein the bacterium species is non-pathogenic to the non-human animal.

8. The method of claim 7 wherein the phage lethal to the bacterium species is lethal to the bacterium species and non-lethal to other bacteria which naturally occur within the digestive tract and wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement;

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host; and

testing the phages which can utilize the bacterium species as a host to determine which of the phages which can utilize the bacterium species as a host are lethal to the bacterium species and also non-lethal to other bacteria which can naturally occur within the digestive tract of the animal.

9. The method of claim 7 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures above 25° C.

10. The method of claim 7 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures of at least 50° C.

11. The method of claim 7 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement;

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host, the growth conditions comprising temperatures of at least 50° C; and

wherein the method further comprises, before inserting the phage into the digestive tract, storing the phage for some period of time at a temperature of 30° C or higher.

12. The method of claim 7 wherein the animal from which the excrement is collected is the same as the animal into which the phage is inserted.

13. The method of claim 7 wherein the excrement is collected from a first animal and inserted into a second animal, the first and second animals belonging to a common genus.

14. The method of claim 7 wherein the excrement is collected from a first animal and inserted into a second animal, the first and second animals belonging to different genera.

15. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising inserting two genetically different phages lethal to the bacterium species into an non-human animal's digestive tract, wherein the bacterium species is non-pathogenic to the non-human animal.

16. The method of claim 15 wherein the two different phages are inserted substantially simultaneously.

17. The method of claim 15 wherein the two different phages are inserted sequentially.

18. A method for selectively reducing a concentration of a bacterium species pathogenic to humans comprising:

inserting a first mixture into an animal's digestive tract, the first mixture comprising a first concentration of a phage lethal to the bacterium species, the bacterium species being non-pathogenic to the animal; and

after inserting the first mixture into the animal's digestive tract, inserting a second mixture into the animal's digestive tract, the second mixture comprising a second concentration of the phage lethal to the bacterium species, the second concentration being greater than the first concentration.

19. A method for reducing a concentration of a bacterium species pathogenic to humans comprising:

applying a phage lethal to the bacterium species on a non-food surface; and

providing a phage from the surface and a bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage.

20. The method of claim 19 further comprising, before applying the phage on the surface, obtaining the phage from an excrement of an animal.

21. The method of claim 19 further comprising, before applying the phage on the surface, obtaining the phage from an excrement of a non-human animal.

22. A method for reducing a concentration of a bacterium species within a meat processing plant comprising applying a phage lethal to the bacterium species to at least one surface within the meat processing plant.

23. The method of claim 1, 7, 19 or 22 wherein the bacterium species is a pathogenic coliform.

24. The method of claim 1, 7, or 22 wherein the bacterium species is a pathogenic form of *Salmonella*.

25. The method of claim 1, 7, 19 or 22 wherein the bacterium species is *E. coli* O157:H7 and wherein the phage is that identified by ATCC number 55952.

26. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract and non-pathogenic to the animal, the method comprising:

collecting excrement from the animal;

obtaining a phage lethal to the bacterium species from the animal excrement;

applying the phage lethal to the bacterium species to a surface; and

providing a phage from the surface and the bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

27. The method of claim 7 or 26 wherein the step of obtaining the phage comprises:

procuring a mix of phages from the animal excrement; and

applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host.

28. The method of claim 26 wherein said surface is on a human food.

29. The method of claim 26 wherein the step of applying the phage to said surface comprises spraying the phage on meat and other surfaces within a meat processing plant.

30. The method of claim 26 wherein the animal is a ruminant, the bacterium species is *E. coli* O157:H7, and the isolated phage is that identified by ATCC number 55952.

31. The method of claim 26 wherein the animal is a ruminant and the bacterium species is a pathogenic strain of *E. coli* O157.

32. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract and non-pathogenic to the animal, comprising:

collecting excrement from the animal;

obtaining a first phage lethal to the bacterium species from the excrement;

obtaining a second phage lethal to the bacterium species from the excrement, the second phage being genetically different than the first phage;

applying the first phage lethal to the bacterium species to a surface;

providing a first phage from the surface and the bacterium pathogenic to humans in proximity to each other to infect the bacterium with the first phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface;

applying the second phage lethal to the bacterium species to the surface; and

providing a second phage from the surface and the bacterium pathogenic to humans in proximity to each other to infect the bacterium with the second phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

33. The method of claim 32 wherein the first phage and the second phage are applied substantially simultaneously to the surface.

34. The method of claim 32 wherein the second phage is applied to the surface after the first phage is applied to the surface.

35. A method for selectively reducing a concentration of a bacterium species pathogenic to humans which is a natural part of the bacterial flora of an animal digestive tract and non-pathogenic to said animal, comprising:

collecting excrement from the animal;

obtaining a phage lethal to the bacterium species from the animal excrement;

applying the first phage lethal to the bacterium species to a surface, the phage being applied to a first concentration;

after applying the phage to a first concentration, reapplying the phage to the surface to a second concentration, the second concentration being greater than the first concentration; and

providing a phage from the surface and the bacterium pathogenic to humans in proximity to each other to infect the bacterium with the phage and to reduce a concentration of the bacterium species pathogenic to humans on said surface.

36. A method for producing meat for human consumption comprising the following steps:

inserting a phage lethal to a bacterium species into a digestive tract of an animal to reduce a concentration of the bacterium species within the digestive tract, the bacterium species being pathogenic to humans and non-pathogenic to the animal; and

slaughtering the animal and producing meat therefrom for human consumption.

37. The method of claim 36 further comprising, after slaughtering the animal, applying the phage lethal to the bacterium species to the meat.

38. The method of claim 7 or 36 wherein the animal is a ruminant and the bacterium species is a pathogenic coliform.

39. The method of claim 7 or 36 wherein the animal is a bird and the bacterium species is a pathogenic form of *Salmonella*.

40. The method of claim 36 further comprising:
collecting animal excrement; and
obtaining the phage lethal to the bacterium species from the excrement.

41. A method of detecting a bacterial strain *in vivo* within an animal comprising:
obtaining a phage which selectively utilizes the bacterial strain as a host;
inserting the phage into the digestive tract of an animal; and
determining if the phage multiplies within the animal.

42. The method of claim 41 wherein the obtaining the phage comprises:
procuring a mix of phages from the animal excrement; and
applying growth conditions to the mix of phages to selectively grow those phages within the mix of phages which can utilize the bacterium species as a host.

43. The method of claim 41 wherein the determining if the phage multiplies within the animal comprises determining an amount of phage excreted by the animal.

44. The method of claim 41 wherein the bacterial strain is *E. coli* O157:H7.

45. A method of detecting a bacterial strain *in vivo* within an animal which comprises, in a common step, both detecting the bacterial strain and reducing a concentration of the bacterial strain within the animal.

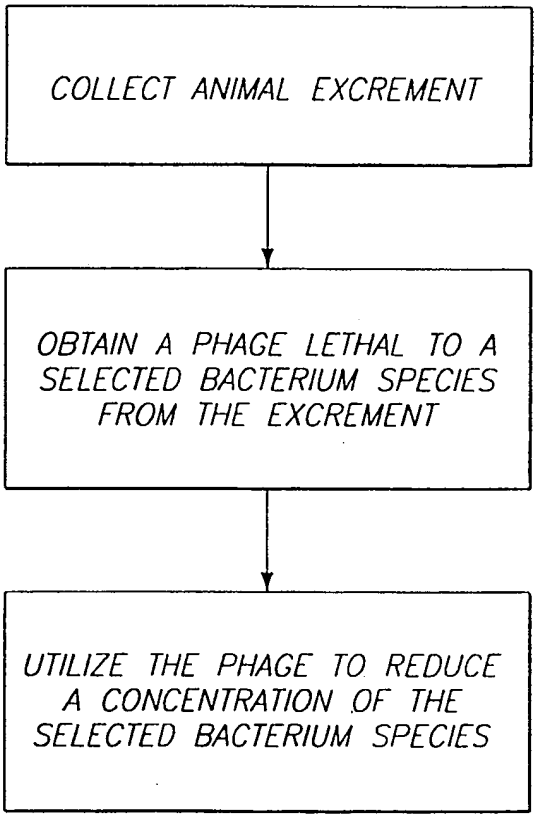

COLLECT ANIMAL EXCREMENT
OBTAIN A PHAGE LETHAL TO A SELECTED BACTERIUM SPECIES FROM THE EXCREMENT
UTILIZE THE PHAGE TO REDUCE A CONCENTRATION OF THE SELECTED BACTERIUM SPECIES

FIG. 1

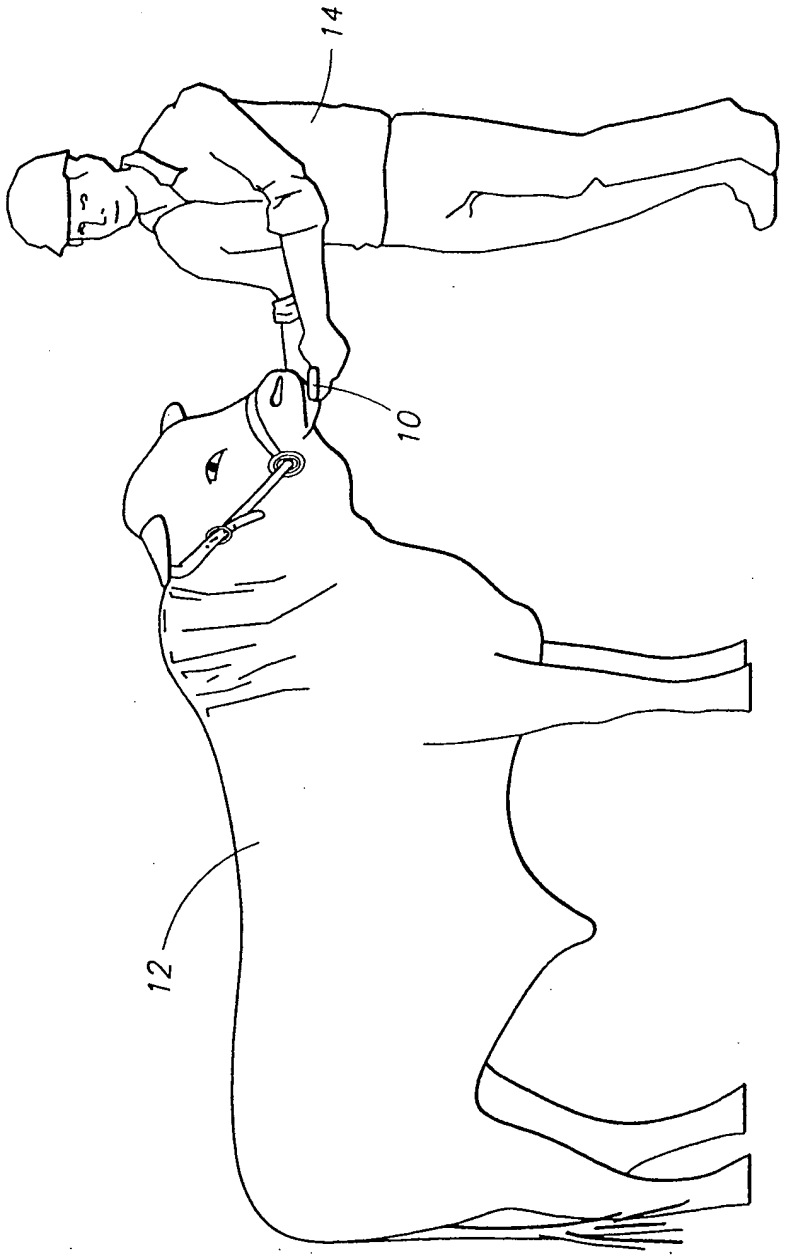
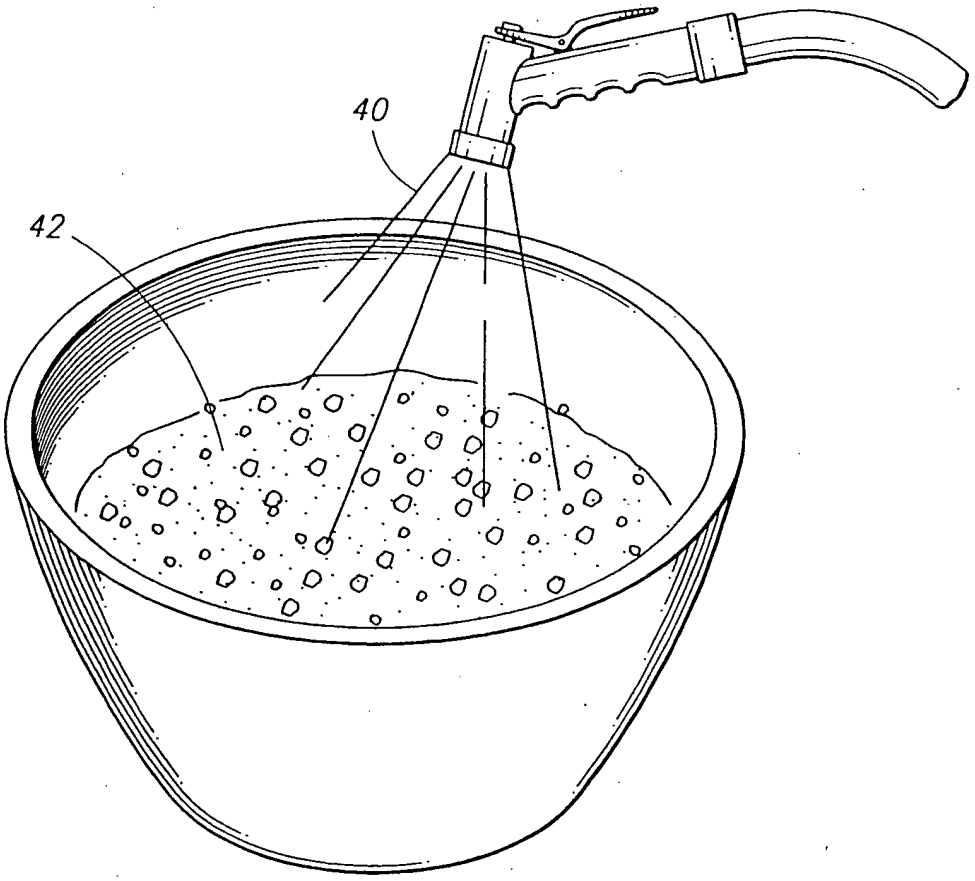


Fig 2



Фиг 3

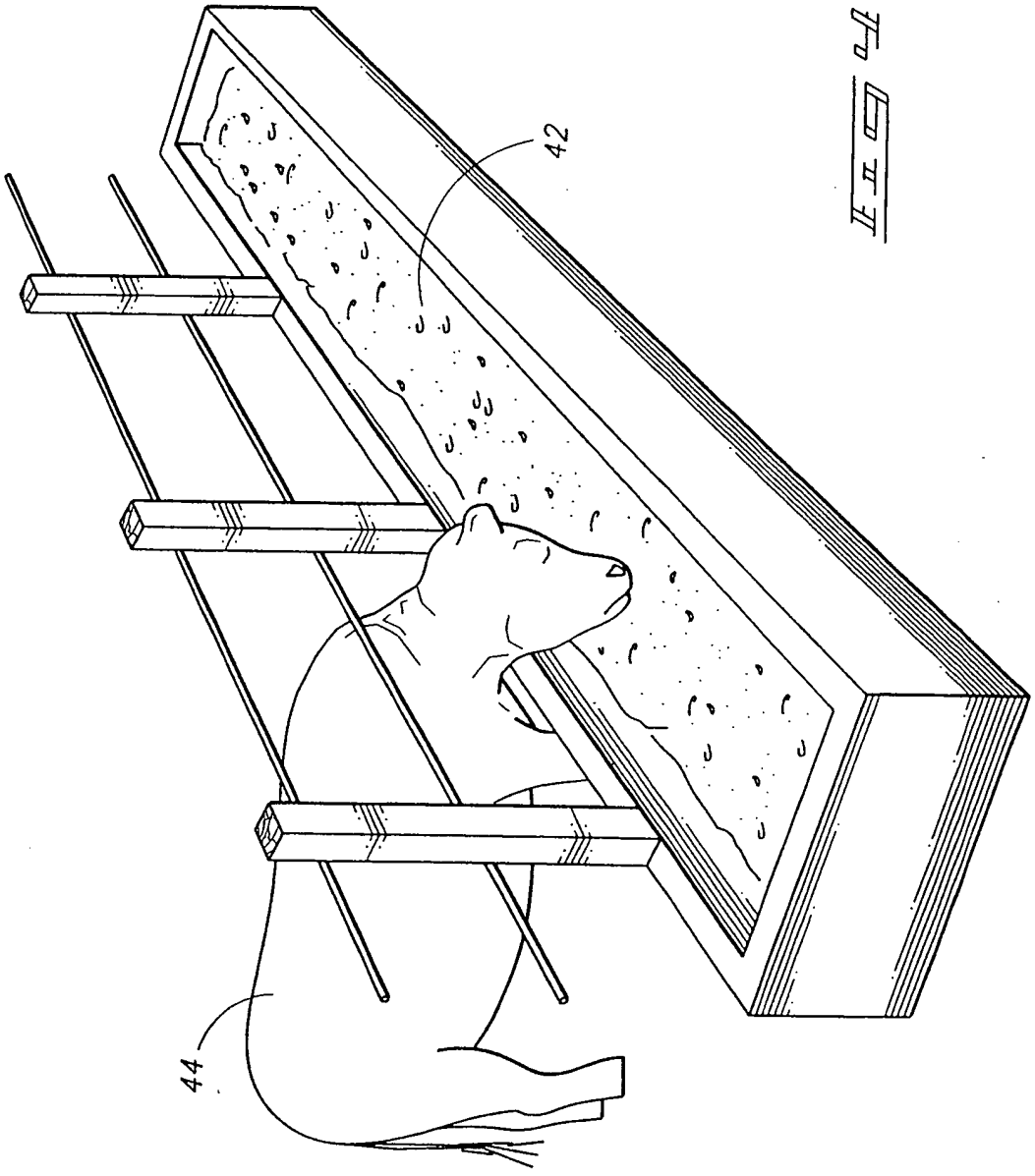


Fig 5

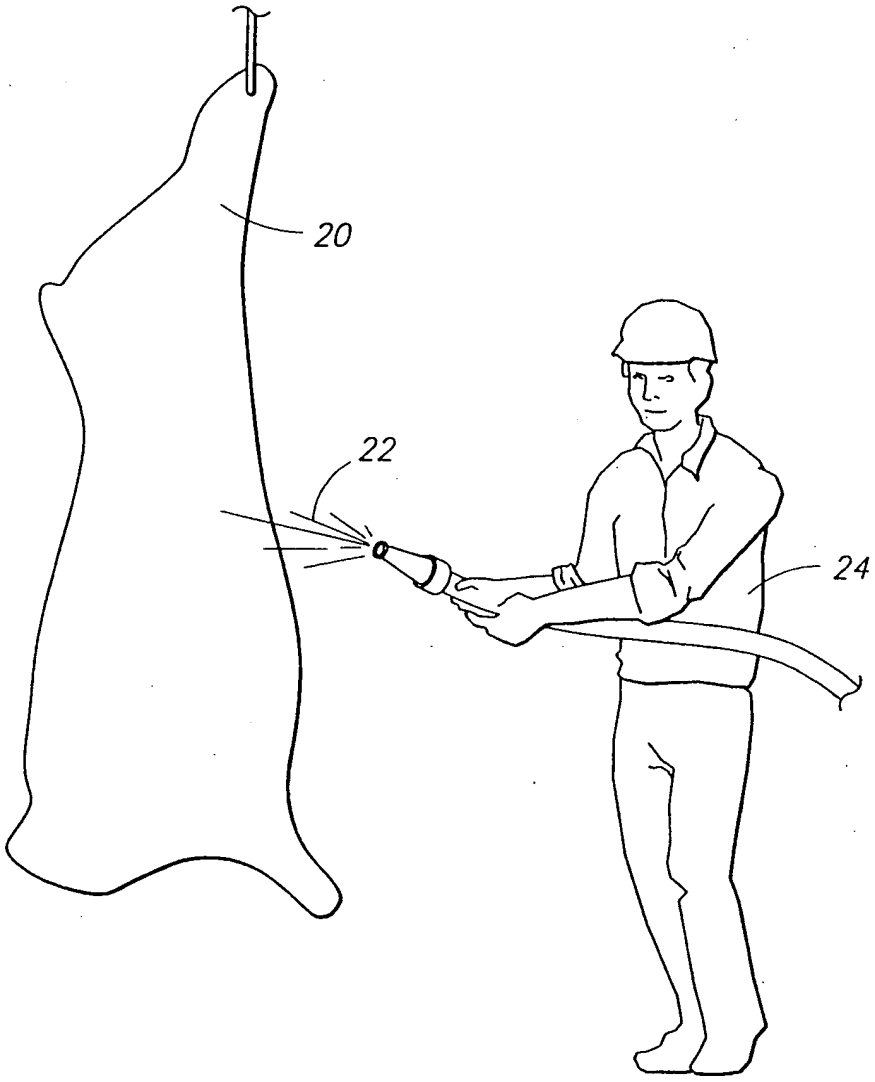


Fig 5

INTERNATIONAL SEARCH REPORT

Int· ional Application No
PCT/US 98/07395

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/76 A23L3/3571 C12N7/00 C12Q1/70 C12Q1/04
//(C12Q1/04,C12R1:92)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K A23L C12N C12Q C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 290 295 A (MICROBIAL DEVELOPMENTS LIMITED) 9 November 1988	1-24,27, 30-35, 38-41, 43-52, 54,56-67
Y	see the whole document	25,26, 28,29, 36,37, 42,53,55
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intr onal Application No
PCT/US 98/07395

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A.B. RONNER ET AL.: "ISOLATION AND CHARACTERIZATION OF A COLIPHAGE SPECIFIC FOR ESCHERICHIA COLI 0157:H7" JOURNAL OF FOOD PROTECTION, vol. 53, no. 11, November 1990, pages 944-947, XP002073167 AMES, IA, US cited in the application	73
Y	see the whole document	25,26, 28,29, 36,37, 42,53,55
X	EP 0 403 292 A (MICROBIAL DEVELOPMENTS LIMITED) 19 December 1990 see page 4, line 35 - line 56; claims	8,63-67
A	WO 88 04326 A (MCDONNELL DOUGLAS CORPORATION) 16 June 1988 see claims 1-3,9,10	68-72
A	EP 0 414 304 A (UNILEVER NV) 27 February 1991 see the whole document	1-72
P,X	WO 98 08944 A (BIO VENTURE BANK CO., LTD.) 5 March 1998 see abstract see page 20, line 11 -& DATABASE WPI Section Ch, Week 9820 Derwent Publications Ltd., London, GB; Class D13, AN 98-230262 XP002072584 & WO 98 08944 see abstract	22,25, 30-32, 37,52, 53,55

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

rnational application No.
PCT/US 98/07395

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-21,32*,33*,39*,50*,51*,55*,61,62 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
(* = partially, as far as being dependent on claims 1 or 9)

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int ional Application No
PCT/US 98/07395

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 290295 A	09-11-1988	WO 9003122 A	05-04-1990
		DE 3873402 A	10-09-1992
		DK 252588 A	08-11-1988
		ES 2051844 T	01-07-1994
		FI 8802129 A	08-11-1988
		GB 2206028 A,B	29-12-1988
		GR 3005371 T	24-05-1993
		IE 62187 B	28-12-1994
		US 4851240 A	25-07-1989
		US 5006347 A	09-04-1991
EP 403292 A	19-12-1990	NONE	
WO 8804326 A	16-06-1988	AU 1043788 A	30-06-1988
		CA 1313111 A	26-01-1993
		EP 0298094 A	11-01-1989
EP 414304 A	27-02-1991	AU 637806 B	10-06-1993
		AU 6110990 A	21-02-1991
		CA 2023556 A	22-02-1991
		DE 69013812 D	08-12-1994
		ES 2065471 T	16-02-1995
		JP 2080412 C	09-08-1996
		JP 3118306 A	20-05-1991
		JP 7103005 B	08-11-1995
WO 9808944 A	05-03-1998	AU 3869297 A	19-03-1998